Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes

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Metal cation homeostasis is essential for plant nutrition and resistance to toxic heavy metals. Many plant metal transporters remain to be identified at the molecular level. In the present study, we have isolated *AtNramp* cDNAs from *Arabidopsis* and show that these genes complement the phenotype of a metal uptake deficient yeast strain, *smf1*. *AtNramp*s show homology to the *Nramp* gene family in bacteria, yeast, plants, and animals. Expression of *AtNramp* cDNAs increases Cd^{2+} sensitivity and Cd^{2+} accumulation in yeast. Furthermore, *AtNramp3* and *AtNramp4* complement an iron uptake mutant in yeast. This suggests possible roles in iron transport in plants and reveals heterogeneity in the functional properties of *Nramp* transporters. In *Arabidopsis*, *AtNramps* are expressed in both roots and aerial parts under metal replete conditions. Interestingly, *AtNramp3* and *AtNramp4* are induced by iron starvation. Disruption of the *AtNramp3* gene leads to slightly enhanced cadmium resistance of root growth. Furthermore, overexpression of *AtNramp3* results in cadmium hypersensitivity of *Arabidopsis* root growth and increased accumulation of Fe, on Cd^{2+} treatment. Our results show that *Nramp* genes in plants encode metal transporters and that *AtNramps* transport both the metal nutrient Fe and the toxic metal cadmium.

Metal cations are essential for plant nutrition (1). Metals such as iron (Fe), manganese (Mn), and copper (Cu) are necessary cofactors for many enzymatic reactions. Some metals such as zinc (Zn) play important structural roles in proteins. Furthermore, metal cations recently have been shown to be involved in signaling in animals (2) and plants (3). However, plants also need to control against excessive accumulation of essential cations and toxic heavy metals, such as cadmium (Cd^{2+}), lead, mercury, and arsenic.

Metal transporters are essential to maintain intracellular metal homeostasis (4). In plants, metal cation transporters play important roles in several steps of metal nutrition. These transport proteins mediate metal uptake in root cells and metal transfer between cells and organs. Metal transporters are involved in metal detoxification by mediating the transport of metal cations or metal chelates from the cytosol to the vacuolar compartment (5–8). A better knowledge of the mechanisms of metal transport in plants is needed for understanding and manipulating plant nutrition and plant resistance to toxic heavy metals.

Uptake of metal cations has been investigated in various species. These studies point to multiple pathways for the low- and high-affinity and constitutive and inducible influx of metal cations, such as Zn and Fe (9–11). It has been suggested that toxic metals such as cadmium enter plant cells by transporters for essential cations, such as Fe and calcium (12–15). For example, in pea, Fe deficiency, which is known to stimulate high-affinity Fe uptake also stimulates Cd^{2+} uptake (12). Recently, screening of plant cDNA libraries for genes able to restore the growth defects of yeast metal transport mutants has led to the identification of plant genes encoding transporters that allow the uptake of Cu (16), Fe, Mn, and Zn (17–19) and various cations (14) in yeast. The ion transport function of these cloned transporters has not yet been directly analyzed in roots.

Genes encoding members of the *Nramp* family of integral membrane proteins were identified through very diverse genetic screens (20). *Nramp1* was cloned in mouse as a locus involved in intracellular bacterial pathogen sensitivity (21). *Nramp* homologous sequences have now been identified in bacteria, fungi, plants, and animals. It was discovered recently that some *Nramp* proteins function as metal transporters. *SMF1*, a yeast *Nramp* homologue, was shown to encode a manganese transporter (22). Then, *DCT1/Nramp2*, a mammalian homologue of the *Nramp* genes, was isolated in a functional screen for Fe transport systems and shown to encode a broad specificity metal transporter (23). *DCT1/Nramp2* was also shown to be a genetic determinant for anemia, suggesting a role in endosomal recycling of Fe (24). *Nramp* genes have been sequenced in some plant species (25) but have not yet been characterized functionally, with the exception of the ethylene insensitivity gene *EIN2*, which functions in signal transduction with no metal transport function (26).

In the present study, we have isolated plant genes homologous to the *Nramp* family in *Arabidopsis*. Furthermore, we have combined heterologous expression studies in yeast and molecular physiological studies in plants to show that *Arabidopsis Nramp* genes encode broad specificity metal transporters that contribute to metal sensitivity in plants. In *Saccharomyces cerevisiae*, *Nramp* gene expression complements the phenotype of yeast mutants impaired in Mn and Fe transport and increases Cd^{2+} sensitivity and Cd^{2+} content in yeast cells. *AtNramp3* mRNA levels are increased on Fe starvation. Characterization of a mutant carrying a T-DNA insertion in *AtNramp3* and overexpression of this gene demonstrate the contribution of this gene to Fe transport and Cd^{2+} sensitivity *in planta*.

Materials and Methods

Cloning of *AtNramp* Genes. *AtNramp1* cDNA was cloned by screening the λYES library of *Arabidopsis thaliana*, ecotype Columbia (27), using an expressed sequence tag clone (dBEST accession no. Z30530) as the probe. *AtNramp3* and *AtNramp4* were amplified by PCR using Advantage *Taq* cDNA polymerase (CLONTECH) from first-strand cDNA prepared from *Arabidopsis* roots using the primers NR1-1S (5′-AATGCCACAACTCGAGAACAACG-3′) and NR1-0R (5′-GAAAGCTAAGAACTCATGATCCTAAGG-3′) for *AtNramp3*, and the primers NR2-3S (5′-GAAATATGTCGGAGACTGATAGAG-

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF202539 and AF202540).

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3′) and NR2-3R (5′-CCTTACTCACTCATCATCCCTC-3′) for *AtNramp4*. The truncated version of *AtNramp3* was generated by PCR using NR1-1S and muNR1-R (5′-CGGGATCCTTACAC-CACAATATATCCTGAACCAGCATATATCTCATTGGA-GAAGAACTCC-3′). The PCR fragments were cloned in pGEMT-easy (Promega) and excised either with *Not*I for cloning in pDR195 or with *Eco*RI for cloning in pMON530 (Monsanto).

Yeast Strains and Growth Conditions. The strains used in this study were INVSc1 (Invitrogen), DEY1453 (17), and *smf1* (*MATα his3 ade2 leu2 trp1 ura3smf1*::URA*3ura3*::*TRP1*). This *smf1* strain was derived from the *smf1* from Supek *et al.* (22) by transformation with linearized pRH530, a plasmid containing *ura3*::*TRP1*, and selection for cells that were trp^+ and ura^-. Yeast cells were grown on yeast extract/peptone/dextrose before transformation and synthetic dextrose-ura after transformation. Yeast cells were transformed according to standard procedures (Invitrogen). The *fet3fet4* (DEY1453) strain was grown in media supplemented with 0.2 mM $FeCl_3$. Complementation of the *smf1* phenotype was tested by using media buffered at pH 6 with 50 mM Mes and supplemented or not with 20 mM EGTA.

Metal Accumulation in Yeast. Yeast cells transformed with empty or *AtNramp* vector were grown for 24 h starting from an OD of 0.01 on synthetic dextrose-ura supplemented with 3 μM Cd^{2+} and $^{109}Cd^{2+}$ at a specific activity of about 4×10^5 cpm/nmol. The cells were harvested on filters and washed twice with 0.1 M $CaCl_2$ before the cell-associated radioactivity was quantified. Short-term uptake experiments were performed as described for Cd^{2+} (14) and for Fe (17).

Insertion Mutant Isolation. For identification of a mutant carrying a T-DNA insertion in *AtNramp3*, superpools of genomic DNA representing 1,000 independent *Arabidopsis* transformants were screened by PCR using Ex-*Taq* polymerase (Takara Shuzo, Kyoto). Four pairs of primers were used, each including an *AtNramp3* gene-specific primer (NR1-1S or NR1-0R) and a T-DNA-specific primer (5′-GATGCACTCGAAATCAGCCAATTTTAGAC-3′ for the left border of the T-DNA and 5′-TCCTTCAATCGTT-GCGGTTCTGTCAGTTC-3′ for the right border of the T-DNA). The PCR product from one superpool (NR1-1S/Left border), which gave a positive signal after southern hybridization with an *AtNramp3* cDNA probe, was reamplified by nested PCR using NR1-2S (5′-CAACGAGCCACTTCTAATCAACG-3′) and a nested primer specific for the left border of the T-DNA (5′-CCGCAATGTGTTATTAAGTTGTCTAAGCG-3′). Sequencing of the nested PCR product revealed that the T-DNA insertion in *AtNramp3* was located between Glu-467 and Val-468. The DNA from the 10 pools of 100 plants from which the positive superpool was constituted were screened by PCR. Then, subpools of 20 plants were screened, leading to the identification of a single subpool containing the *AtNramp3* disruption mutant. Finally, PCR screening of DNA samples from individual plants lead to the identification of two homozygous and one heterozygous plant carrying the insertion in the *AtNramp3* gene. Their genotypes were confirmed by genomic Southern blot after digestion with *Eco*RI that yields a 7-kb fragment in the wild type and a 4-kb fragment in the mutant.

Gene Expression. *Arabidopsis* seedlings were grown for 10 days vertically on plates containing Murashige and Skoog medium with 1% sucrose and 1% agar A (Sigma). Total RNA was extracted from about 60 seedlings by using the Trizol reagent (Life Technologies, Grand Island, NY). For Northern blots 15 μg total RNA was separated on denaturing 1.2% formaldehyde agarose gel, transferred to a Hybond-N nylon membrane (Amersham Pharmacia), and hybridized with probes made from full-length *AtNramp* cDNAs. For RT-PCR analysis, after digestion of the genomic DNA, the first cDNA strand from *AtNramp3* was synthesized from 10 μg of total RNA extracted from wild-type, control T-DNA plants, or *AtNramp3* mutant. A complementary oligonucleotide located 5′ of the insertion on AtNramp3 cDNA (NR1-RT: 5′-GAGAAGAACTCCAA-CAAAAGATAACC-3′) was used for reverse transcription. Subsequently the reverse transcription product was amplified using NR1-1S and NR1-RT primers.

Cd^{2+} Sensitivity of Root Growth. *Arabidopsis* seedlings were grown vertically on plates containing a minimal medium without microelements (2.5 mM H_3PO_4/5 mM KNO_3/2 mM $MgSO_4$/1 mM $Ca(NO_3)_2$/1 mM Mes adjusted with KOH to pH 5.5/1% sucrose/1% agar A). This medium was supplemented with various concentrations of Cd^{2+}. The plants to be compared were grown on the same plate. After 3 days of stratification at 4°C, the seedlings were grown for 10 days before measurement of the root length. A Student's *t* test was performed to evaluate the significance of the differences observed.

Metal Ion Content Measurements. Two hundred seeds were germinated in a flask containing 50 ml of the minimal medium used for the root growth assays (without agar) and grown for 10 days on a shaker at 100 rpm. The plants were harvested by vacuum filtration and washed with 10 ml $CaCl_2$ (0.1 M) and with 10 ml EDTA (10 mM). The dry weight (D.W.) of the samples was measured after drying overnight at 60°C. Subsequently, the samples were digested in 0.2–0.4 ml of 70% HNO_3 (trace metal grade; Fisher) for 3 days, and complete digestion was achieved by heating the samples to 100°C for 30 min. After dilution, the metal content of the samples was determined by inductively coupled plasma optical emission spectroscopy. The results are given in ppm (mg metal/kg D.W.)

Results

Identification and Cloning of *Arabidopsis Nramp* Genes. A search for sequences with homology to yeast and animal *Nramp* genes led to the identification of a family of *Nramp* homologous genes in *Arabidopsis*. *AtNramp1* was identified as an expressed sequence tag sequence (212P17T7), and its cDNA was deposited later in the database first as *AtNramp1* (AF165125), together with *AtNramp2* (AF141204), and then as *PMIT1* (AF181687). *AtNramp3*, *AtNramp4* and, during the completion of this work, *AtNramp5* and *AtNramp6* were identified as genomic sequences on BACs T20D16, K8K14, F28A21, and T24D18, respectively. In addition, the sequence of *EIN2* (AF 141202), a locus controlling ethylene sensitivity in *Arabidopsis* that shares homology with *Nramp* genes (26), was recently deposited in the database. The predicted *EIN2* protein differs from *AtNramp1* to *AtNramp5* in that it includes a large hydrophilic C-terminal domain (26). *Arabidopsis Nramp* genes encode a family of highly hydrophobic membrane proteins. *AtNramp2*, *AtNramp3*, *AtNramp4*, and *AtNramp5* are most closely related sharing between 67% and 75% conserved amino acid sequences, whereas *AtNramp1* is more distantly related with only 33–37% amino acid conservation (Fig. 1). The Nramp domain within the EIN2 protein is even more divergent and shares only 17–20% identity at the amino acid level with *AtNramp 1* to *AtNramp 5* (Fig. 1). We cloned three *AtNramp* genes from *Arabidopsis*. A full-length *AtNramp1* cDNA (AF 165125) was cloned by screening an *Arabidopsis* cDNA library using the expressed sequence tag. *AtNramp3* (AF202539) and *AtNramp4* (AF202540) were cloned by RT-PCR using the data from the genome sequencing project and sequenced.

***Arabidopsis Nramp* Genes Complement Yeast Mutants Defective in Mn and Fe Transport.** *SMF1*, a yeast *Nramp* homologue, has been shown to be a manganese transporter (22) with a broad metal specificity (28, 29). As a first step to investigate *AtNramp* function, we tested whether the plant *AtNramp* cDNAs are able

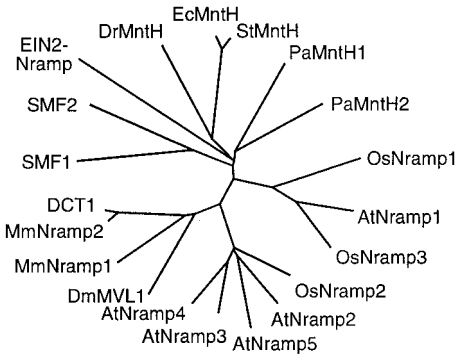

Fig. 1. Phylogenetic tree of the *Nramp* gene family in animals, fungi, and plants. *Nramp* homologous genes: *AtNramp1* (AF 165125), *AtNramp2* (AF 141204), *AtNramp3* (AF202539), *AtNramp4* (AF202540), and *AtNramp5* from *Arabidopsis*; *OsNramp1* (L41217), *OsNramp2* (L81152), and *OsNramp3* (U60767) from rice (*Oryza sativa*). *SMF1* (U15929) and *SMF2* (U00062) from yeast (*Saccharomyces cerevisiae*). *MmNramp1* (L13732) and *MmNramp2* (L33415) from mouse (*Mus musculus*). *DCT1* is the rat homologue of *MmNramp2*. *DmMVL1* stands for *Mavolio 1* (U23948) from *Drosophila melanogaster*. DrMntH (AE002012), EcMntH (AF161318), PaMntH1 (AF161319), PaMntH2 (AF161320), and StMntH (AF161317) from bacteria (*Deinococcus radiodurans*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*). The phylogenetic tree of *Nramp* sequences was drawn by using the TREEVIEW program after comparison of the deduced protein sequences with the CLUSTALX program.

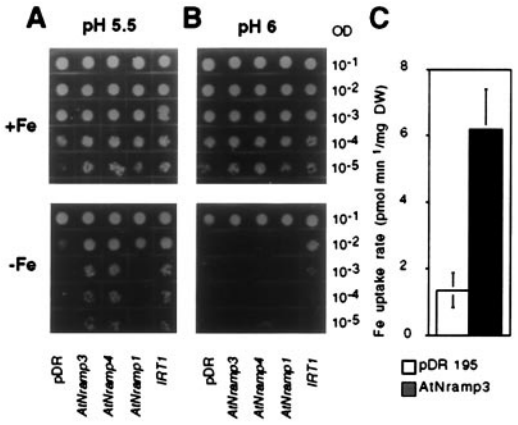

Fig. 3. *AtNramp3* and *AtNramp4* complement a Fe uptake deficient yeast mutant. Growth of *fet3fet4* yeast cells expressing *AtNramp1*, *AtNramp3*, or *AtNramp4* or *IRT1* on synthetic dextrose-URA (pH 5.5) (*A*) or (pH 6) (*B*) supplemented 0.2 mM $FeCl_3$ (*Upper*) or without added Fe (*Lower*). *fet3fet4* yeast cells were transformed with the empty pDR195 vector, with pDR195 containing the cDNA of *AtNramp1*, *AtNramp3*, or *AtNramp4*, or with the vector pFL61 containing the *IRT1* cDNA. Transformed strains were grown overnight in liquid synthetic dextrose-ura supplemented with 0.2 mM $FeCl_3$. The cultures were diluted to ODs of 10^{-1} to 10^{-5} (as indicated) and spotted on synthetic dextrose-ura plates. The plates were incubated at 30°C for 3 days before photography. (*C*) FeII uptake rates in *fet3fet4* yeast expressing *AtNramp3* or transformed with the empty vector. FeII accumulation was measured after 10 min in 30 μM ^{55}Fe at pH 5.5.

to complement the phenotype of a *S. cerevisiae* strain disrupted in the *SMF1* gene (*smf1*). *smf1* yeast fail to grow on synthetic medium containing high concentrations of the divalent cation chelator EGTA. Fig. 2 shows that expression of *AtNramp1*, *AtNramp3*, and *AtNramp4* under the control of a constitutive yeast promoter restored the growth of *smf1* yeast on synthetic medium containing 20 mM EGTA, whereas a strain transformed with the empty vector pDR195 or *EIN2* could not grow, as reported (26). Thus, *AtNramp1*, *AtNramp3*, and *AtNramp4* complement the phenotype of *smf1* yeast. These data demonstrate that *AtNramp1*, *AtNramp3*, and *AtNramp4* cDNAs encode functional proteins and provide initial evidence that these proteins can mediate manganese transport.

Subsequently, we tested whether the *AtNramp* genes are able to complement a yeast Fe transport mutant. The *fet3fet4* yeast strain is defective in both low- and high-affinity Fe uptake systems (30), requires high Fe concentrations in its medium for growth, and shows reduced Fe uptake. Expression of *AtNramp3* or *AtNramp4* restored the ability of *fet3fet4* to grow on a medium not supplemented with Fe (Fig. 3*A*). *AtNramp3* or *AtNramp4* complemented the phenotype of *fet3fet4* as efficiently as *IRT1*, a well-characterized Fe uptake transporter gene from *Arabidopsis* (Fig. 3*A*; ref. 17), whereas *AtNramp1* was less efficient. Interestingly, the ability of *AtNramp3* or *AtNramp4* to complement *fet3fet4* was sharply pH dependent; whereas, at pH 5.5, *AtNramp3* or *AtNramp4* expression complemented the growth phenotype of *fet3fet4* yeast as efficiently as *IRT1* (Fig. 3*A*); at pH 6, only *IRT1* was still able to complement Fe uptake (Fig. 3*B*). The pH dependence of the complementation correlates with the finding that the mammalian homologue *Nramp2/DCT1* functions as a H^+-metal symporter (23). When we compared FeII uptake rates in *fet3fet4* yeast transformed with the empty vector or expressing *AtNramp3*, we found that *AtNramp3* expression consistently increased the Fe uptake rate at pH 5.5 (Fig. 3*C*). On average, the uptake rate was 6.4 times higher in *AtNramp3*-expressing yeast cells. These results provide additional evidence that *AtNramp3* and *AtNramp4* are metal transporters and reveal functional differences between the different members of the *Arabidopsis Nramp* family: *AtNramp3* and *AtNramp4*, which are closely related, complement *fet3fet4*, whereas *AtNramp1*, which is evolutionary more distant, is less efficient.

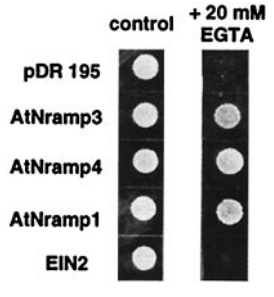

Fig. 2. AtNramp genes complement a yeast mutant deficient in Mn uptake. Growth of *smf1* yeast cells expressing *AtNramp1*, *AtNramp3*, or *AtNramp4* on SD plates supplemented (*Right*) or not (*Left*) with 20 mM EGTA. *smf1* yeast cells were transformed either with empty pDR195 or with pDR195 containing the cDNAs of *AtNramp1*, *AtNramp3*, *AtNramp4* or of *EIN2*. Transformed strains were grown overnight in liquid synthetic dextrose-ura. The cultures were diluted to an OD of 0.1 and spotted on plates containing synthetic dextrose-ura (pH 6). The plates were incubated at 30°C for 3 days before photography.

***AtNramp* Expression Enhances Cd^{2+} Sensitivity and Increases Cd^{2+} Content in Yeast.** Cadmium is a toxic heavy metal transported across plant membranes by physiological metal transporters (14, 15). Therefore, we tested the influence of *AtNramp* expression on Cd^{2+} toxicity in yeast. On plates containing synthetic medium supplemented with 10 μM Cd^{2+}, the growth of wild-type yeast expressing *AtNramp1*, *AtNramp3*, and *AtNramp4* was strongly impaired compared with yeast transformed with the empty vector (Fig. 4*A*). In liquid culture, 3 μM Cd^{2+} strongly reduced the growth of yeast expressing *AtNramp1*, *AtNramp3*, and *AtNramp4* compared with the control. Measurement of the Cd^{2+} content after 24 h showed that Cd^{2+} inhibition of growth by *AtNramp*s correlates with increased Cd^{2+} accumulation (Fig. 4*B*). However, when Cd^{2+} uptake was studied in short-term experiments (20 min), expression of *AtNramp3* or *AtNramp4* did

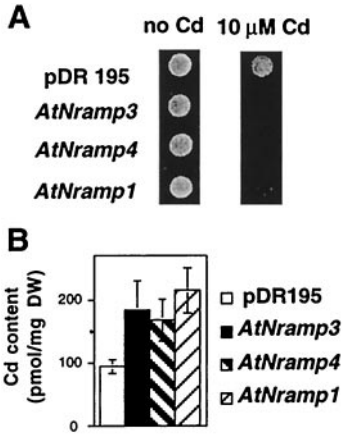

Fig. 4. *AtNramp* expression increases Cd^{2+} sensitivity and Cd^{2+} content in yeast. (*A*) Growth of INVSc1 yeast cells expressing *AtNramp1*, *AtNramp3*, or *AtNramp4* on plates containing synthetic dextrose-ura buffered to pH 6 with 50 mM Mes without $CdCl_2$ (*Left*) or supplemented with 10 μM $CdCl_2$ (*Right*). The plates were incubated at 30°C for 3 days before photography. (*B*) Cd^{2+} content of INVSc1 yeast cells expressing *AtNramp1*, *AtNramp3*, or *AtNramp4* grown for 24 h in liquid synthetic dextrose-ura supplemented with 3 μM Cd^{2+}, as measured by the uptake of $^{109}Cd^{2+}$. INVSc1 yeast cells were transformed either with the empty pDR195 vector, or with pDR195 containing the *AtNramp1*, *AtNramp3*, or *AtNramp4* cDNA. Transformed strains were grown overnight in synthetic dextrose-ura. The cultures were then spotted on plates or inoculated in 10 ml synthetic dextrose-ura at an OD of 0.01. Error bars represent the SE from four independent experiments ($P < 0.03$, 0.06, and 0.07 for *AtNramp1*, *AtNramp3*, and *AtNramp4*, respectively).

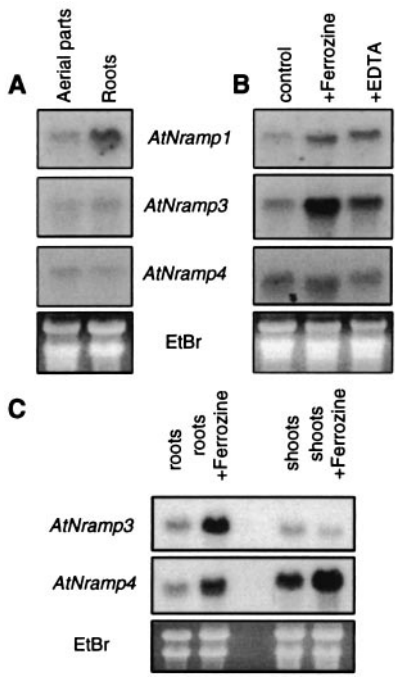

Fig. 5. Differential induction of *AtNramps* by Fe starvation. (*A*) Expression of *AtNramp1*, *AtNramp3*, or *AtNramp4* in roots and aerial parts of *Arabidopsis* seedlings. RNA was extracted separately from roots and aerial parts (cotyledons, hypocotyls, and primary leaves). (*B*) Effect of Fe or metal starvation on *AtNramp1*, *AtNramp3*, or *AtNramp4* expression in whole *Arabidopsis* seedlings. (*C*) On Fe starvation, *AtNramp3* and *AtNramp4* are up-regulated in roots. RNA was extracted from seedlings grown on (MS) or on MS supplemented with either 1 mM ferrozine or 1 mM EDTA. *Arabidopsis* were grown on MS medium for 10 days before RNA extraction. Then, 15 μg of total RNA was loaded in each lane and transferred on a nylon membrane. The membrane was probed with full-length DNA probes corresponding to *AtNramp1*, *AtNramp3*, or *AtNramp4*. Ethidium bromide staining of the gel is shown as a loading control (EtBr).

not increase Cd^{2+} accumulation (data not shown), indicating a low rate of enhanced Cd^{2+} accumulation.

***AtNramp* Gene Expression in *Arabidopsis* Seedlings.** We examined the expression pattern of *AtNramp* genes. When *Arabidopsis* seedlings were grown on metal replete Murashige and Skoog medium, *AtNramp1*, *AtNramp3*, and *AtNramp4* mRNAs could be detected by Northern blot in total RNA extracts from both roots and aerial parts of *Arabidopsis* seedlings (Fig. 5*A*). *AtNramp1* was preferentially expressed in roots, whereas *AtNramp3* and *AtNramp4* were expressed at comparable levels in roots and aerial parts (Fig. 5*A*).

We further tested whether *AtNramp* gene expression could be enhanced by metal starvation. For this purpose, *Arabidopsis* seedlings were grown on media supplemented with 1 mM EDTA, a divalent cation chelator, or 1 mM ferrozine, a specific chelator of Fe^{2+}. *AtNramp1* and *AtNramp3* expression were significantly increased when seedlings were grown on 1 mM EDTA, and this increase was even more obvious for *AtNramp3* when they were grown on 1 mM Ferrozine (Fig. 5*B*, $n = 4$). Furthermore, we showed that the up-regulation of *AtNramp3* mRNA by Fe starvation is root-specific (Fig. 5*C*), whereas *AtNramp4* mRNA levels were increased in both roots and shoots by Fe starvation (Fig. 5 *B* and *C*, $n = 3$ of 4 experiments). We chose to focus initial molecular physiological studies in *Arabidopsis* on *AtNramp3*, which complements a yeast mutant deficient in Fe transport and is up-regulated by Fe starvation.

***AtNramp3* Disruption Leads to Moderate Cd^{2+} Resistance in *Arabidopsis* Seedlings.** To investigate the physiological function of *AtNramp3* in plants, we used PCR to screen a population of *Agrobacterium*-transformed *Arabidopsis* for individuals carrying a T-DNA insertion in this gene. A plant with a T-DNA insertion in the 3′ end of the *AtNramp3* gene was identified (Fig. 6*A*). Both PCR and genomic Southern experiments confirmed the mutant genotype. After a backcross with wild-type *Arabidopsis*, segregation of the kanamycin resistance phenotype indicated that the original plant carried a T-DNA insertion at a single locus. RT-PCR experiments using primers 5′ of the insertion site allowed the detection of *AtNramp3* mRNA in wild-type but not in mutant plants, suggesting that the truncated gene was either not transcribed or that its mRNA was unstable. The insertion results in the truncation of the last 42 C-terminal amino acids of the *AtNramp3* protein and replacement by 10 amino acids encoded by the left border of the T-DNA before reaching a stop codon. The truncated cDNA corresponding to this mutant gene failed to complement the *smf1* yeast strain phenotype (Fig. 6*B*).

When grown in soil or in minimal or Murashige and Skoog medium, the mutant did not display any clearly visible phenotype. However, detailed analyses of root growth on plates containing 1–30 μM Cd^{2+} showed that root growth in the mutant was moderately but significantly more resistant to Cd^{2+} than the control (Fig. 6*C*). For example, the roots of the *AtNramp3-1* mutant were 30.5 ± 13.0% longer than controls on 1 μM Cd^{2+} ($n = 4$ experiments, $P < 0.004$) and 57.0 ± 9.5% longer than controls on 10 μM Cd^{2+} ($n = 5$, $P < 0.002$; Fig. 6*C*). Thus, *AtNramp3* contributes to Cd^{2+} toxicity in *Arabidopsis*. Measurements of the metal content in whole plants using inductively coupled plasma optical emission spectroscopy did not show any significant difference in Fe, Mn, and Zn content and Cd^{2+} accumulation between controls and mutants under the imposed conditions (data not shown). It is possible that the differences in metal accumulation between mutant and control were too small to be resolved at the whole plant level. For example, *AtNramp3* disruption might alter metal levels in specific tissues, cell types, or cell compartments.

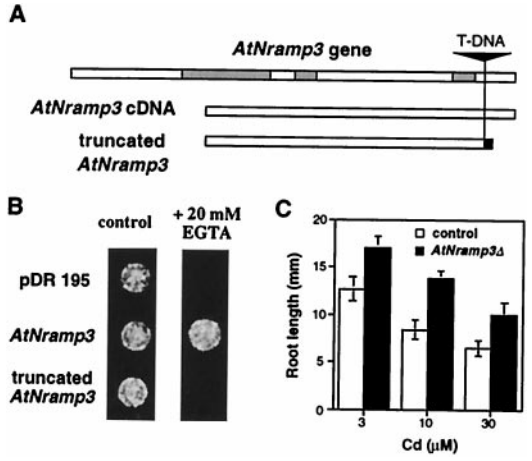

Fig. 6. Characterization of a mutant carrying a T-DNA insertion in *AtNramp3*. (*A*) Localization of the T-DNA insertion site in the *AtNramp3* gene and cDNA. Gray boxes represent introns, black box represents T-DNA sequence putatively translated in the mutant. (*B*) The T-DNA disrupted allele of *AtNramp3* cannot complement *smf1*, a Mn uptake deficient yeast mutant. *smf1* yeast cells were transformed with empty pDR195, pDR195 containing the cDNA of *AtNramp3*, or a truncated *AtNramp3* cDNA corresponding to the predicted mRNA product of the disrupted allele of *AtNramp3*. (*C*) Root growth shows increased resistance to Cd^{2+} in *AtNramp3-1* mutant. Root length of *Arabidopsis* seedlings from control with a T-DNA insertion outside *AtNramp3* (open bars) or mutant with T-DNA insertion in *AtNramp3* (filled bars) grown vertically for 10 days on plates containing minimal medium supplemented with various concentrations of Cd^{2+}. The length of the roots grown on plates without Cd^{2+} were 52.0 ± 5.0 mm for the control and 56.2 ± 2.4 mm for the *AtNramp3*Δ mutant. The graph represents the results of one representative experiment of four. Values are means of 12–16 root lengths, and error bars represent SE.

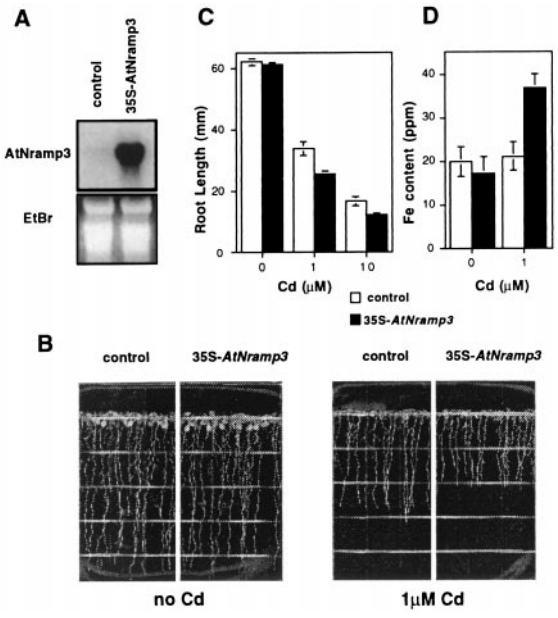

Fig. 7. Overexpression of *AtNramp3* confers Cd^{2+} hypersensitivity in *Arabidopsis*. (*A*) Northern blot analysis of an overexpressing line. Conditions are as described in Fig. 5. (*B*) Root growth hypersensitivity to Cd^{2+}. *Arabidopsis* seedlings were grown vertically for 10 days on plates containing minimal medium supplemented (*Right*) or not (*Left*) with 1 μM Cd^{2+}. (*C*) Root length of *Arabidopsis* seedlings grown vertically for 10 days on plates containing minimal medium supplemented with 0, 1, or 10 μM Cd^{2+}. The graph represents the results of one representative experiment of four with one of the two lines analyzed in detail. Values are mean of measurements on 12–16 roots, and error bars represent SE. (*D*) Iron over-accumulation in ^{35}S-*AtNramp3* plants on Cd^{2+} treatment. *Arabidopsis* seedlings were grown for 10 days in liquid minimal medium supplemented or not with 1 μM Cd^{2+}. The Fe content was quantified by inductively coupled plasma optical emission spectroscopy. The mean ± SE of three independent experiments is represented. Control (open bars): homozygous, single insertion line transformed with empty pMON530. ^{35}S-*AtNramp3* (filled bars): homozygous, single insertion line transformed with pMON530 containing *AtNramp3* cDNA driven by the ^{35}S CaMV promoter.

Overexpression of *AtNramp3* Leads to Cd^{2+} Hypersensitivity and Fe Overaccumulation. To further test a role of *AtNramp3* in *Arabidopsis*, we generated plants ectopically overexpressing this gene. The *AtNramp3* cDNA was placed under the strong constitutive CaMV35S promoter and introduced into *Arabidopsis* through *Agrobacterium*-mediated transformation. Two independent lines with elevated *AtNramp3* mRNA levels (Fig. 7*A*) carrying single T-DNA insertions were selected and self-crossed to obtain stable homozygous overexpressing lines. Both lines displayed Cd^{2+} hypersensitivity as assayed by root growth measurements (Fig. 7 *B* and *C*). On Cd^{2+}-free medium, the root length was the same in overexpressing lines and a control line transformed with the empty vector, pMON530 (0.8 ± 2.4%, $n = 4$, $P > 0.3$). However on media containing 1 and 10 μM Cd^{2+}, the root length of the overexpressing plants was reduced by 24.4 ± 6.5% ($n = 3$, $P < 0.007$) and 31.0 ± 3.0% ($n = 4$, $P < 0.004$), respectively. The phenotype was maintained from the T3 to T4 generation as a genetically inheritable trait. This result confirms that *AtNramp3* can modulate heavy metal toxicity in plants.

To determine whether *AtNramp3* affects Fe accumulation, we measured the iron content of control and *AtNramp3* overexpressing seedlings grown in minimal medium supplemented or not with 1 μM Cd^{2+}. *AtNramp3* overexpressing plants accumulated 77 ± 15% ($n = 3$ experiments) more Fe than control plants transformed with empty vector, when exposed to Cd^{2+} (Fig. 7*D*). In contrast, no increase in Cd^{2+} content or reproducible change of the whole plant content in other metals such as Mn and Zn could be detected under these conditions. These findings reveal a role for *AtNramp3* in Fe transport in *Arabidopsis*.

Discussion

We have cloned three members of the *Arabidopsis Nramp* gene family: *AtNramp1*, *AtNramp3*, and *AtNramp4*. Heterologous expression in yeast points to a role of *AtNramp* proteins in transition metal transport: *AtNramp* genes are able to complement yeast mutants altered in Mn and Fe uptake. Furthermore, expression of *AtNramp* genes in yeast leads to an increase in Cd^{2+} sensitivity and Cd^{2+} accumulation. Investigation of *AtNramp* expression as well as manipulation of these genes *in planta* also points to a role in metal homeostasis in plants: *AtNramp3* and *AtNramp4* are induced by Fe starvation; disruption of *AtNramp3* in *Arabidopsis* reduces Cd^{2+} sensitivity of root growth, and, conversely, overexpression of *AtNramp3* leads to an increase in Cd^{2+} sensitivity and a conditional increase in Fe accumulation.

The *Nramp* homologues *SMF1* in yeast and *DCT1/Nramp2* in mammals mediate the uptake of a broad range of metals (22, 23, 28, 29). However, no evidence for metal transport was found for other genes that belong to the *Nramp* family or contain *Nramp*-like domains. Neither *Nramp1* from mice, which encodes the natural resistance macrophage associated protein and determines sensitivity to mycobacterium infection such as tuberculosis or leprosy (21), nor the recently isolated *Nramp* homologue *EIN2*, which is a determinant of ethylene sensitivity in plants (26), can complement a yeast mutant disrupted in the *Nramp* homologue *SMF1* (26, 31). In contrast, the mouse *Nramp2* and the three *AtNramp* genes that we have cloned are able to rescue *smf1* yeast growth (31). Although not all *Nramp* proteins seem to function as metal transporters, these results provide initial data suggesting that the members of the *AtNramp* family in *Arabidopsis* function as metal transporters.

Possible Roles of *AtNramp3* and *AtNramp4* as Iron Transporters in Plants. Although it has been proposed that *Nramps* may function as broad specificity transition metal transporters (23, 28), the mammalian homologue *Nramp2/DCT1* has been more specifically implicated in Fe homeostasis (24). *AtNramp3* and *AtNramp4* complement the phenotype of *fet3fet4*, a yeast mutant deficient in Fe uptake, as effectively as the well-characterized *Arabidopsis* Fe uptake transporter *IRT1* (17). Furthermore, *AtNramp3* mediates FeII uptake in yeast. Our data lead to a model in which two families of Fe transporters, *IRT*s and *AtNramp*s, may contribute to Fe homeostasis *in planta*. Iron transport by *AtNramp3* and *AtNramp4* was stimulated by extracellular acidification, suggesting that the contribution of *AtNramp* and *IRT* transporters to physiological Fe transport may vary depending on pH.

Physiological Functions of *AtNramps* in Plants. Expression studies show that *AtNramp* genes are expressed in both roots and aerial parts of *Arabidopsis* seedlings under metal replete conditions. In rice, *OsNramp3* is expressed primarily in roots, and *OsNramp2* in leaves, whereas *OsNramp3* is present in both (25). The expression of plant *Nramps* is distinct with respect to the Fe uptake transporter gene of the ZIP family, *IRT1*, which is expressed specifically in roots in response to metal starvation (17). The expression of *AtNramp* suggests a contribution of *Nramp* transporters to constitutive metal transport in plants, whereas *IRT1* has been proposed to function as an inducible metal uptake system in roots (17).

Interestingly, *AtNramp1* and *AtNramp3* mRNA levels are increased by metal starvation, and *AtNramp3* and *AtNramp4* are strongly up-regulated by iron starvation. Therefore, the increased accumulation of Mn and Cd observed on Fe starvation in pea and *Arabidopsis* (12, 19) might be because of the induction of both the multispecific transport system *IRT1* (12, 17, 19) and *Nramp* transporters.

The induction of *AtNramp3* on Fe starvation suggests a possible role of *AtNramp3* in inducible Fe uptake. The *Atnramp3-1* insertion mutant did not display a clearly visible phenotype on low Fe minimal medium or rich medium supplemented with the Fe chelating molecule Ferrozine. This is possibly because of functional redundancy with other inducible Fe transport systems, such as *IRT1*. However, the role of *AtNramp3* in Fe transport *in planta* is supported by the observation that *AtNramp3* overexpressing plants contained elevated levels of Fe, on Cd^{2+} treatment. It is possible that this phenotype could be revealed in the presence of Cd^{2+} because Cd^{2+} blocks other Fe transporters, such as *IRT1* (17, 19). Together, these data indicate a contribution of *AtNramp3* to Fe transport in plants.

Disruption of the *AtNramp3* gene leads to a moderate increase in Cd^{2+} resistance. Considering that *AtNramp3* has at least four closely related homologues in the *Arabidopsis* genome, it is remarkable that knocking out a single *Nramp* gene leads to an observable phenotype. In agreement with the phenotype of the mutant with a disrupted *AtNramp3*, overexpression of this gene confers increased Cd^{2+} sensitivity in *Arabidopsis*. In conclusion, the phenotype of plants disrupted or overexpressing *AtNramp3* point to a role of *AtNramp*s in physiological Cd^{2+} transport and Cd^{2+} sensitivity in plants.

In conclusion, we have functionally characterized three members of the *Nramp* gene family in *Arabidopsis*. Our data provide evidence that *AtNramp* genes encode multispecific metal transport systems in plants that can transport Fe, Mn, and Cd^{2+}. Analyses of the expression of *AtNramp* genes in *Arabidopsis* suggest that all these *Nramp* genes play roles in constitutive metal transport. Furthermore, the complementation of *fet3fet4* yeast mutant by *AtNramp3* together with the induction of *AtNramp3* gene expression by Fe starvation and the increased accumulation of Fe in *AtNramp3* overexpressing seedlings on Cd^{2+} treatment suggest that *AtNramp3* plays a role in inducible Fe transport in plants.

Note Added in Proof: Another paper describing the molecular characterization of *AtNramp1* and *AtNramp2* is soon to be published (32).

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